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TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER	
RDID01046US	
U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	
09/890202	
INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE / PRIORITY DATE CLAIMED

TITLE OF INVENTION

METHOD FOR PREPARING ENDOTOXIN-FREE NUCLEIC ACIDS OR NUCLEIC ACIDS WITH REDUCED ENDOTOXIN CONTENT AND THE USE THEREOF

APPLICANT(S) FOR DO/EO/US

GRIMM, Stefan; and NEUDECKER, Frank

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
4. ☐ The US has been elected by the expiration of 19 months from the priority date (Article 31).
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   a. ☒ is attached hereto (required only if not communicated by the International Bureau).
   b. ☐ has been communicated by the International Bureau.
   c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
   a. ☒ is attached hereto.
   b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
7. ☐ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   a. ☐ are attached hereto (required only if not communicated by the International Bureau).
   b. ☐ have been communicated by the International Bureau.
   c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   d. ☐ have not been made and will not be made.
8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☒ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☒ A copy of the International Search Report (PCT/ISA/210).

Items 13 to 20 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☒ A **FIRST** preliminary amendment.
16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
17. ☐ A substitute specification.
18. ☐ A change of power of attorney and/or address letter.
19. ☐ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
22. ☒ Certificate of Mailing by Express Mail
23. ☒ Other items or information:

General Appointment of Representative for U.S. Patent and Trademark Office Matters; and Return postcard.

[redacted] TION NO. (IF KNOWN, SEE 37 CFR 09/890202 | INTERNATIONAL APPLICATION NO. | ATTORNEY'S DOCKET NUMBER RDID01046US

JC17 Rec'd PCT/PTO 26 JUL 2001

24. The following fees are submitted:.	CALCULATIONS PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :	
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO **$1000.00**	
☒ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO **$860.00**	
☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO **$710.00**	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) **$690.00**	
☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) **$100.00**	
ENTER APPROPRIATE BASIC FEE AMOUNT =	**$860.00**
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).	**$0.00**

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE	
Total claims	- 20 =	0	x $18.00	**$0.00**
Independent claims	- 3 =	0	x $80.00	**$0.00**
Multiple Dependent Claims **(check if applicable)**.			☐	**$0.00**

TOTAL OF ABOVE CALCULATIONS =	**$860.00**
☐ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.	**$0.00**
SUBTOTAL =	**$860.00**
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +	**$0.00**
TOTAL NATIONAL FEE =	**$860.00**
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) **(check if applicable).** ☐	**$0.00**
TOTAL FEES ENCLOSED =	**$860.00**
	Amount to be: refunded $
	charged $

a. ☐ A check in the amount of _____ to cover the above fees is enclosed.

b. ☒ Please charge my Deposit Account No. **50-0877** in the amount of **$860.00** to cover the above fees. A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **50-0877.** A duplicate copy of this sheet is enclosed.

d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Kenneth J. Waite
Roche Diagnostics Corporation
9115 Hague Road, Bldg. D
P.O. Box 50457
Indianapolis, IN 46250-0457
Telephone No.: (317) 521-3104
Facsimile No.: (317) 521-2883

SIGNATURE

Kenneth J. Waite
NAME

45,189
REGISTRATION NUMBER

July 26, 2001
DATE

Docket No. RDID 01046 US

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of: Grimm et al.

Application No.: To Be Assigned Group No.: To Be Assigned

Filed: July 26, 2001 Examiner: To Be Assigned

For: METHOD FOR PREPARING ENDOTOXIN-FREE NUCLEIC ACIDS OR NUCLEIC ACIDS WITH REDUCED ENDOTOXIN CONTENT AND THE USE THEREOF

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Sir:

Please enter the following amendments prior to examination of the above-referenced application:

IN THE CLAIMS:

Please enter the following amendments to claims 1 through 16 as filed in the originally filed application. Both a clean and a marked-up copy of the claims as amended are attached.

Respectfully submitted,

Date: July 26, 2001

Kenneth J. Waite, Reg. No. 45,189
Roche Diagnostics Corporation
9115 Hague Road, Bldg. D
P.O. Box 50457
Indianapolis, IN 46250-0457
Telephone No.: (317) 521-3104
Facsimile No.: (317) 521-2883

[Patent claims]

We claim:

1. (Amended) A method for isolating and purifying nucleic acids and/or oligonucleotides from a biological sample, [characterized in that] said method comprising:

   - disruption of the biological sample[is disrupted,] and removal of protein components and other insoluble components [are removed,] with the fraction not containing said protein and insoluble components being a residue,

   - addition of an aqueous solution of potassium acetate [is added] to the residue and removal of non-soluble components [are removed],

   - mixing and incubation of the potassium acetate-containing solution [is mixed and incubated] with an alcoholic solution containing a detergent,

   - obtaining the supernatant [obtained is] and contacting and incubating said supernatant [contacted and incubated] with a silica gel-like support material, and

   - isolating the purified nucleic acids and/or oligonucleotides [are isolated] from the soluble fraction.

2. (Amended) The method as claimed in claim 1, wherein [characterized in that] the alcoholic solution is a mixture of isopropanol with an ionic detergent.

3. (Amended) The method as claimed in claim 1 [or 2], wherein [characterized in that] the alcoholic solution contains one or more ionic detergents at a concentration of 0.5 to 10%

(w/v) in 100% strength alcohol.

4. (Amended) The method as claimed in claim 1, [any of claims 1 to 3,] wherein [characterized in that] an aqueous solution containing 1 to 6 M potassium acetate is used.

5. (Amended) The method as claimed in claim 4, wherein [characterized in that] the aqueous solution contains 2 to 4 M potassium acetate.

6. (Amended) The method as claimed in claim 1, [any of claims 1 to 5,] wherein [characterized in that] the silica gel-like support material used is a suspension of silicon dioxide.

7. (Amended) The method as claimed in claim 1, [any of claims 1 to 6,] wherein [characterized in that] the silica gel-like support material is [rewashed] washed at least once with acetone.

8. (Amended) The method as claimed in claim 1, [any of claims 1 to 7,] wherein [characterized in that] plasmid DNA with an endotoxin content of less than 100 U/µg is obtained.

9. (Amended) The method as claimed in claim 8, wherein [characterized in that] the endotoxin content is not more than 10 U/µg of plasmid DNA.

10. (Amended) A nucleic acid or oligonucleotide comprising [an endotoxin-free nucleic acid or oligonucleotide or] a nucleic acid or oligonucleotide with reduced endotoxin content obtainable according to a method as claimed in any of claims 1 to 9.

11. (Amended) A method of using [The use of] nucleic acids and/or oligonucleotides [obtained according to any of the

methods as claimed in any of claims 1 to 9 for] comprising transfecting eukaryotic or prokaryotic cells, wherein the nucleic acids and/or oligonucleotides are obtained according to any one of the methods claimed in claims 1 to 9.

12. (Amended) A method of using [The use of] nucleic acids and/or oligonucleotides [obtained according to any of the methods as claimed in any of claims 1 to 9 for] comprising producing an agent for the treatment of genetic disorders, wherein the nucleic acids and/or oligonucleotides are obtained according to any one of the methods claimed in claims 1 to 9.

13. (Amended) A kit [composition] comprising the following components:

- at least one solution suitable for the disruption of a biological sample,
- an aqueous potassium acetate solution,
- a solution of detergent/alcohol, and
- a silica gel-like support material.

14. (Amended) The kit [composition] as claimed in claim 13, wherein [characterized in that] the [following] components are [included]:

- a solution suitable for alkaline lysis of biological sample material,
- a salt solution containing 1 to 6 M potassium acetate,
- an alcoholic solution containing 0.5 to 10% (w/v) SDS in 100% strength isopropanol and
- a silica gel-like support material.

15. (Amended) The kit [composition] as claimed in claim 13 [or 14], characterized in that the support material included is a suspension of silicon dioxide.

16. (Amended) A method of [The use of potassium acetate for] isolating, purifying and/or separating [endotoxin-free nucleic acids and/or oligonucleotides or] nucleic acids and/or oligonucleotides comprising mixing a pre-purified biological sample lysate with potassium acetate, wherein such method results in the isolation, purification and/or separation of nucleic acids and/or oligonucleotides with reduced endotoxin content when compared to [from and of, respectively, a] the pre-purified biological sample.

We claim:

1. A method for isolating and purifying nucleic acids and/or oligonucleotides from a biological sample, said method comprising:

   - disruption of the biological sample and removal of protein components and other insoluble components with the fraction not containing said protein and insoluble components being a residue,

   - addition of an aqueous solution of potassium acetate to the residue and removal of non-soluble components,

   - mixing and incubation of the potassium acetate-containing solution with an alcoholic solution containing a detergent,

   - obtaining the supernatant and contacting and incubating said supernatant with a silica gel-like support material, and

   - isolating the purified nucleic acids and/or oligonucleotides from the soluble fraction.

2. The method as claimed in claim 1, wherein the alcoholic solution is a mixture of isopropanol with an ionic detergent.

3. The method as claimed in claim 1, wherein the alcoholic solution contains one or more ionic detergents at a concentration of 0.5 to 10% (w/v) in 100% strength alcohol.

4. The method as claimed in claim 1, wherein an aqueous solution containing 1 to 6 M potassium acetate is used.

5. The method as claimed in claim 4, wherein the aqueous solution contains 2 to 4 M potassium acetate.

6. The method as claimed in claim 1, wherein the silica gel-like support material used is a suspension of silicon dioxide.

7. The method as claimed in claim 1, wherein the silica gel-like support material is washed at least once with acetone.

8. The method as claimed in claim 1, wherein plasmid DNA with an endotoxin content of less than 100 U/µg is obtained.

9. The method as claimed in claim 8, wherein the endotoxin content is not more than 10 U/µg of plasmid DNA.

10. A nucleic acid or oligonucleotide comprising a nucleic acid or oligonucleotide with reduced endotoxin content obtainable according to a method as claimed in any of claims 1 to 9.

11. A method of using nucleic acids and/or oligonucleotides comprising transfecting eukaryotic or prokaryotic cells, wherein the nucleic acids and/or oligonucleotides are obtained according to any one of the methods claimed in claims 1 to 9.

12. A method of using nucleic acids and/or oligonucleotides comprising producing an agent for the treatment of genetic disorders, wherein the nucleic acids and/or oligonucleotides are obtained according to any one of the methods claimed in claims 1 to 9.

13. A kit comprising the following components:
- at least one solution suitable for the disruption of a

biological sample,

- an aqueous potassium acetate solution,
- a solution of detergent/alcohol, and
- a silica gel-like support material.

14. The kit as claimed in claim 13, wherein the components are:
    - a solution suitable for alkaline lysis of biological sample material,
    - a salt solution containing 1 to 6 M potassium acetate,
    - an alcoholic solution containing 0.5 to 10% (w/v) SDS in 100% strength isopropanol and
    - a silica gel-like support material.

15. The kit as claimed in claim 13, characterized in that the support material included is a suspension of silicon dioxide.

16. A method of isolating, purifying and/or separating nucleic acids and/or oligonucleotides comprising mixing a pre-purified biological sample lysate with potassium acetate, wherein such method results in the isolation, purification and/or separation of nucleic acids and/or oligonucleotides with reduced endotoxin content when compared to the pre-purified biological sample.

Roche Diagnostics GmbH

Method for preparing endotoxin-free nucleic acids or nucleic acids with reduced endotoxin content and the use thereof

The invention relates to a method for isolating and purifying nucleic acids and/or oligonucleotides from a biological sample, to the use of the isolated or purified nucleic acid and/or oligonucleotide for transfecting cells and also for the production of an agent for the treatment of genetic disorders, to a composition suitable for the isolation or purification method and also to the use of potassium acetate and a silica gel-like support material for isolating endotoxin-free nucleic acids and/or oligonucleotides or nucleic acids and/or oligonucleotides with reduced endotoxin content.

The quality of isolated nucleic acids is becoming increasingly important. Highly pure nucleic acid fractions, i.e. fractions from which, if possible, all other cell components such as, for example, endotoxins, have been removed, play a central part in gene therapy or in transfecting cells of eukaryotic or also prokaryotic origin. Consequently, in the past few years methods or measures which allow the isolation of nucleic acids from biological sample material with high purity have increasingly been published. The established methods essentially make use of the use of affinity and/or anion exchange chromatography materials and also of ionic detergents or also diluted solutions of higher alcohols. For example, according to WO95/21177 the fractions of interest are subjected to an affinity chromatography or a chromatography on an inorganic solid phase, the latter preferably in the presence of a non-ionic detergent, in order to remove endotoxins and are then further purified by means of anion exchange chromatography. A two-stage chromatography method of this kind, however, is time-

and material-consuming and therefore is more academically valuable. According to another method (WO95/21178) a complicated anion exchange chromatography is likewise absolutely necessary in order to remove residues of a complex salt solution added beforehand.

Furthermore, it has been known for some time that DNA plasmids from complex biological samples of eukaryotic or prokaryotic origin can be isolated by binding to silica gel in the presence of chaotropic salts such as, for example, guanidine hydrochloride (M.A. Marko et al., Analyt. Biochem. 121, (1982) 382-287; EP 0 389 063). However, these methods are not suitable for obtaining low-endotoxin or endotoxin-free nucleic acid fractions. Thus it has been possible to show, for example, that the measures according to Marko et al. (1982) lead to an endotoxin content of more than 10,000 U per µg of DNA. Such an endotoxin-rich DNA fraction is unsuitable for transfecting cells in applications of gene therapy.

It was therefore the object of the invention to provide a method for preparing endotoxin-free nucleic acids or nucleic acids with reduced endotoxin content, as a result of which the disadvantages of established methods, such as in particular complicated column materials, are avoided.

The object is achieved by a method for isolating and purifying nucleic acids and/or oligonucleotides from biological samples, in which the particular biological sample is disrupted, undissolved cell components are resuspended in an aqueous potassium acetate solution, optionally present insoluble components are removed, for example by centrifugation, and the aqueous phase is mixed and incubated with an alcoholic solution containing a detergent. The solution is then contacted with a silica gel-like support material, the aqueous

phase is, if possible, quantitatively removed from the support material binding the nucleic acids or oligonucleotides, for example by suction or centrifugation, and the support material with the DNA is then washed adequately. The washing solution used may be an alcoholic solution or acetone which has proved particularly advantageous. Depending on the volume of the starting sample, an incubation time for contacting the support material of from 10 to not more than 40 minutes at room temperature is sufficient; according to the invention, approx. 20 minutes are normally sufficient.

The skilled worker in principle knows silica gel-like support materials. According to the invention, a suspension of silicon dioxide has proved particularly suitable. A silicon oxide suspension which was prepared by adding acid (e.g. hydrochloric acid) to an aqueous suspension of silicon dioxide and was then autoclaved is particularly suitable for the method of the invention.

The aqueous potassium acetate solution contains potassium acetate preferably in a concentration range from approx. 1 to 6 mol/l, and a range from 2 to 4 mol/l and a weakly acidic pH (approx. pH 4.5-6.8) have resulted, according to the invention, in a particularly high quality of the nucleic acids.

Another advantageous embodiment of the method of the invention is to add to the sample, after addition of the potassium acetate solution, additionally one or more RNA-digesting enzymes such as, for example, RNAse A and/or RNAse T1. In particular for relatively large preparations it has proved advantageous to add the RNA-digesting enzyme(s) in the same medium/buffer in which the potassium acetate salt had been added before. Alternatively, and this is particularly true for relatively small mixtures, the RNA-digesting enzymes

can also be added even during disruption of the biological sample, i.e. together with the lysis buffer (e.g. together with buffer (1 in example 1.2). If a plurality of RNA-digesting enzymes is added, said enzymes may be present in any ratios or else in equal parts. The final concentration of RNA-digesting enzymes in said solution is normally up to or at approx. 150 µg/ml; but even higher enzyme concentrations have not had an adverse effect on the method of the invention.

Normally, according to the invention, an incubation with the potassium acetate solution of from 5 to 10 minutes at 4°C, where appropriate initially at room temperature, is already sufficient for the enzymatic digestion; depending on the amount of sample material used, however, the incubation may be extended accordingly.

Suitable alcoholic solutions according to the invention are in particular high percentage solutions of higher alcohols such as isopropanol. According to the invention, it has proved particularly advantageous if the alcoholic solution is not diluted with water, that is to say virtually 100% of it consists of the particular alcohol, and it additionally contains one or more ionic detergents, at a concentration of 0.5 to 10% (w/v). A 100% isopropanol solution containing approx. 1 to 4% (w/v) SDS has proved particularly suitable according to the invention.

The biological sample can in principle be disrupted or pre-purified according to methods known to the skilled worker. According to the invention, preference is given to alkaline lysis measures, in particular in the case of bacterial host cells. In this way it is possible to remove protein components and other soluble components before contacting the residue which essentially contains nucleic acid components and other non-soluble

cell components with the potassium acetate solution or the alcohol/detergent solution.

Using the method of the invention it is possible to obtain nucleic acids such as, for example, plasmid DNA in high quality, i.e. in particular with an endotoxin content of less than 100 U/µg of DNA, normally of not more than 10 U/µg of DNA.

In particular it must be regarded as surprising that the DNA can be bound with high efficiency to the adsorption matrix after alkaline lysis without the need for the addition of chaotropic substances as described in the prior art. The absence of added chaotropic substances leads to substantial improvements and purifications in the subsequent DNA purification procedure and/or in the corresponding transfection of target cells, that is for cells of both eukaryotic and prokaryotic origin.

Moreover, the endotoxin-free nucleic acids and/or oligonucleotides or the nucleic acids and/or oligonucleotides with reduced endotoxin content, which are obtainable according to the method of the invention, are suitable for producing agents for the treatment of genetic diseases.

The invention further relates to means or compositions for obtaining plasmid DNA from appropriate host cells, which can be, for example, microtiter plates or blocks which may, where appropriate, contain mini columns for purifying plasmid DNA.

The compositions of the invention essentially contain an aqueous potassium acetate solution and also a detergent-containing alcoholic solution and a silica gel-like support material. Moreover, it is advantageous if a solution suitable for disrupting a biological sample, in particular for alkaline lysis, is present. In particular embodiments of the composition the salt

concentration in the potassium acetate solution is in a range from approx. 1 to 6 M, particularly preferably from approx. 2 to 4 M in a weakly acidic medium (pH approx. 4.5-6.8), the alcoholic solution contains isopropanol with approx. 0.5 to 10% (w/v) of an ionic detergent such as, for example, SDS and/or the support material is an aqueous suspension of silicon dioxide.

Figure 1

Endotoxin (lipopolysaccharide, LPS) content in various DNA plasmid fractions after acetone washing ((c),(d)) and SDS precipitation ((b),(d)). The plasmid DNA was isolated by binding to silicon oxide and subsequently washed with isopropanol ((a),(b)) or acetone ((c),(d)), with or without LPS precipitation in the presence of SDS (2.5% in isopropanol). The LPS content was determined colorimetrically, according to the manufacturer's instructions (Boehringer Ingelheim, Germany).

(a) isopropanol/without SDS,
(b) isopropanol/with SDS,
(c) acetone/without SDS,
(d) acetone/with SDS

The following examples further illustrate the invention:

1.1 Cell culture and transfection

Baby hamster kidney (BHK) cells were cultured in DMEM (Dulbecco's Modified Eagle's Medium) supplemented with 5% fetal calf serum (Sigma, Deisenhofen, Germany) in a humidified 5% CO_2 atmosphere. For transfections, the cells were applied to 24-well plates and transfected with 2 µg of plasmid DNA according to the calcium phosphate coprecipitation method as described by Roussel et al. (Mol. Cell. Biol. 4 (1984), 1999-2009). For this purpose, 25 µl of DNA solution were mixed with

25 µl 2 x HBS: 274 mM NaCl, 10 mM KCl, 40 mM HEPES, 1.4 mM Na_2PO_4, pH 6.9 at 4°C in a 96-well plate using a 12-channel pipette (Eppendorf, Hamburg, Germany). After adding 20 µl of a 0.25 M $CaCl_2$ solution (4°C) and mixing, 38 µl were added to the cells after incubation at room temperature for 25 min.

Appropriate aliquots were inoculated in 900 µl of TB medium in wells of 96-well blocks (Qiagen, Hilden, Germany) and cultured with shaking at 300 rpm for approx. 30 hours (37°C). After identification of a positive pool, the DNA was again transfected to confirm the result. The remaining DNA was used to transform bacteria for large-scale plasmid isolation.

1.2 Plasmid isolation with columns

96-well blocks (Qiagen, Hilden, Germany) with bacteria were centrifuged at 3000 g (Sigma centrifuges, Osterode am Harz, Germany) for 5 min. The supernatant was decanted and the blocks were inverted and put on absorbent paper towel for 2 to 3 min. Then 170 µl of buffer P1 (50 mM Tris-HCl/10 mM EDTA pH 8.0, 4°C) were added and the bacteria pellets were resuspended by complete vortex treatment for 10 to 20 min. After addition of 170 µl of buffer P2 (200 mM NaOH, 1% SDS), the block was sealed with foil, inverted and incubated at room temperature for 5 min. The lysis was stopped by adding 170 µl of 4°C cold buffer P3 (3 M potassium acetate pH 5.5, 4°C). Then 10 µl of RnaseA solution (1.7 mg/ml) were added, followed by incubation at room temperature and then at -20°C for 5 min and another centrifugation at 6000 rpm for 10 min. The supernatant was decanted into new blocks and 100 µl of buffer P4 (2.5% (w/v) SDS in isopropanol) were added. The block was subjected to vortexing for 5 min and incubated initially at 4°C for 15 min and then at 20°C for 15 min. The blocks were centrifuged at 6000 rpm for 10 min and the supernatant was [lacuna] into an array of 96

columns (Qiagen) in appropriately cut 96-well plates, had been prepared. These plates were placed in vacuum chambers (Qiagen). Then 150 µl of silicon oxide suspension were added followed by incubation at room temperature for 20 min (the silicon oxide suspension was prepared by adding 150 µl of HCl (37%) to 250 ml of a suspension of 50 mg/ml SiO_2 (Sigma) and subsequent autoclaving).

After applying reduced pressure, the columns were washed twice with 600 µl of acetone (-20°C). The 96-well column plate was put on a 96-well microtiter plate and centrifuged at 6000 rpm for 4 min. The column plate was dried initially at 37°C for 5 min and then in a vacuum chamber for 5 min and then put on another microtiter plate. 70 µl of double-distilled H_2O (60°C) were added followed by centrifugation at 6000 rpm for 3 min. The microtiter plate was stored at -20°C.

1.3 Plasmid isolation without columns

Up to the addition of buffer P4, the method was carried out as described under point 1.2. After centrifugation at 6000 rpm for 10 min, the supernatant was then provided to 96-well POM-microtiter blocks (POM= polyoxymethylene) and 150 µl of silicon oxide suspension were added followed by incubation at room temperature for 20 min. The plates were centrifuged at 6000 rpm for 5 min. The supernatant was carefully decanted and 400 µl of acetone (-20°C) were added. The plates were again vortexed (30 sec) and centrifuged at 6000 rpm for 3 min. This acetone washing was repeated once. The plates were dried initially at room temperature for 5 min and then in a vacuum chamber for 5 min. The pellets were resuspended in 75 µl of water (60°C) and centrifuged at 6000 rpm and 4°C for 10 min. The supernatant was stored in a 96-well microtiter plate at -20°C.

2. Results

Plasmid DNA was isolated from the bacteria cultures using mini columns (see point 1.2). A corresponding protocol without columns is described under point 1.3.

It is important for the transfection step to obtain plasmid DNA of very high purity. For this purpose, silicon dioxide was used as binding matrix for plasmid DNA. Binding of DNA and silicon dioxide in the presence of chaotropic substances is well known (Vogelstein and Gillespie, Proc. Natl. Acad. Sci. USA 76 (1979), 615-619). Surprisingly, however, it was found that even in the absence of an added chaotropic substance such as, for example, guanidine hydrochloride, the plasmid DNA binds to silicon dioxide with sufficient capacity. After subsequent washing in acetone, where appropriate with the addition of SDS, plasmid DNA in excellent quality, corresponding to a purification via a cesium chloride gradient, could be obtained. Commonly, about 10 µg of plasmid DNA with an OD260/280 of greater than 1.8 were obtained from 900 µl of LB medium, 90% of which were present in supercoiled form.

3. Comparison with prior art

Experiment A: Bacteria culture: *E.coli* HB101 pCMVbetaSportGAL, OD_{680}/ml approx. 3.3

In duplicate mixtures, 1.8 ml each of bacteria culture were worked up using the High Pure plasmid isolation kit (Boehringer Mannheim, Cat. No. 1 754 777), which contains a glass-like support material and a strongly chaotropic salt and 1.8 ml each of bacteria culture were processed according to the method of the invention.

The result is as follows:

Yield OD_{260}nm:	Endotoxin content (LAL assay)
High Pure 1: 9.0 µg/100 µl of endotoxin-free water	214 EU/µg of plasmid
High Pure 2: 8.6 µg/100 µl of endotoxin-free water	240 EU/µg of plasmid
Invention 1: 11.00 µg/100 µl of endotoxin-free water	1.41 EU/µg of plasmid
Invention 2: 10.35 µg/100 µl of endotoxin-free water	4.65 EU/µg of plasmid

Procedure according to the method of the invention using a High Pure filter tube:

The bacteria culture was centrifuged at 13,000 rpm for 30 sec and the supernatant was removed.

The cell pellet of 1.8 ml of bacteria culture was further treated as follows:

1. Resuspending in 250 µl of 50 mM Tris-HCl/10 mM EDTA, 100 µg of RNase (DNase-free), pH 8.0, 4°C.

2. Adding 250 µl of 0.2 M NaOH, 1% SDS and 5-10 x inverting the vessel, 5 min at room temperature.

3. Adding 250 µl of 3 M K acetate (4°C) and 5-10 x inverting the vessel, incubating on ice for 5 min.

4. Centrifuging in a bench-top centrifuge at maximum speed for 10 min (14,000 rpm), removing the supernatant and adding 0.2 vol. (approx. 150 µl) of 2.5% SDS in isopropanol (e.g. 7 ml of isopropanol and 1 ml of 20% SDS) and vortexing briefly, incubating at 4°C for 15 min and then incubating at -20°C for 15 min.

5. Centrifuging in a bench-top centrifuge at maximum speed for 10 min (14,000 rpm), removing supernatant.

6. Pipetting supernatant into High Pure filter tube and incubating at room temperature for 20 min.

7. Centrifuging in a bench-top centrifuge at maximum speed for 30 sec (14,000 rpm), discarding the flow-through and washing the filter tube 2 x with 700 µl of ice-cold acetone (centrifuging between the washing steps at 14,000 rpm for 30 sec).

8. After the last washing step, centrifuging again at 14,000 rpm for 30 sec in order to dry the fleece.

9. Eluting DNA by adding 100 µl of endotoxin-free water and incubating at room temperature for 10 min. The DNA is obtained by centrifuging at maximum centrifugation speed for 30-60 sec.

Experiment B: Bacteria culture: *E.coli* JM109pCMVbetaSportGal OD_{580}/ml 2.37

Sample	Method	Modification	Yield [µg/ 100µg]	Endotoxin [EU/µg]
1 and 2	High Pure		9.3/9.3	371.7
3 and 4	High Pure	Incubated on fleece for 20 min; incubated before elution for 10 min	12.8/12.2	2.18
5 and 6	Invention	Without incubations	12.2/12.6	0.63

Result:

- The method of the invention shows approx. 100 fold reduction in endotoxin.

- Furthermore, the inventive method with rapid passing through by centrifugation gives the same yield as using incubation on fleece, and therefore the purification time can now be stated as approx. 70 min. In addition, the inventive method with rapid passing through by centrifugation shows a lower endotoxin value than after incubation on fleece.

Patent claims

1. A method for isolating and purifying nucleic acids and/or oligonucleotides from a biological sample, characterized in that

   - the biological sample is disrupted, protein components and other insoluble components are removed,

   - an aqueous solution of potassium acetate is added to the residue and non-soluble components are removed,

   - the potassium acetate-containing solution is mixed and incubated with an alcoholic solution containing a detergent,

   - the supernatant obtained is contacted and incubated with a silica gel-like support material, and

   - the purified nucleic acids and/or oligonucleotides are isolated from the soluble fraction.

2. The method as claimed in claim 1, characterized in that the alcoholic solution is a mixture of isopropanol with an ionic detergent.

3. The method as claimed in claim 1 or 2, characterized in that the alcoholic solution contains one or more ionic detergents at a concentration of 0.5 to 10% (w/v) in 100% strength alcohol.

4. The method as claimed in any of claims 1 to 3, characterized in that an aqueous solution containing 1 to 6 M potassium acetate is used.

5. The method as claimed in claim 4, characterized in that the solution contains 2 to 4 M potassium acetate.

6. The method as claimed in any of claims 1 to 5, characterized in that the silica gel-like support material used is a suspension of silicon dioxide.

7. The method as claimed in any of claims 1 to 6, characterized in that the silica gel-like support material is rewashed with acetone.

8. The method as claimed in any of claims 1 to 7, characterized in that plasmid DNA with an endotoxin content of less than 100 U/µg is obtained.

9. The method as claimed in claim 8, characterized in that the endotoxin content is not more than 10 U/µg of plasmid DNA.

10. An endotoxin-free nucleic acid or oligonucleotide or a nucleic acid or oligonucleotide with reduced endotoxin content obtainable according to a method as claimed in any of claims 1 to 9.

11. The use of nucleic acids and/or oligonucleotides obtained according to any of the methods as claimed in any of claims 1 to 9 for transfecting eukaryotic or prokaryotic cells.

12. The use of a nucleic acid and/or oligonucleotides obtained according to any of the methods as claimed in any of claims 1 to 9 for producing an agent for the treatment of genetic disorders.

13. A composition comprising the following components:

- at least one solution suitable for the disruption of a biological sample,
- an aqueous potassium acetate solution,
- a solution of detergent/alcohol, and
- a silica gel-like support material.

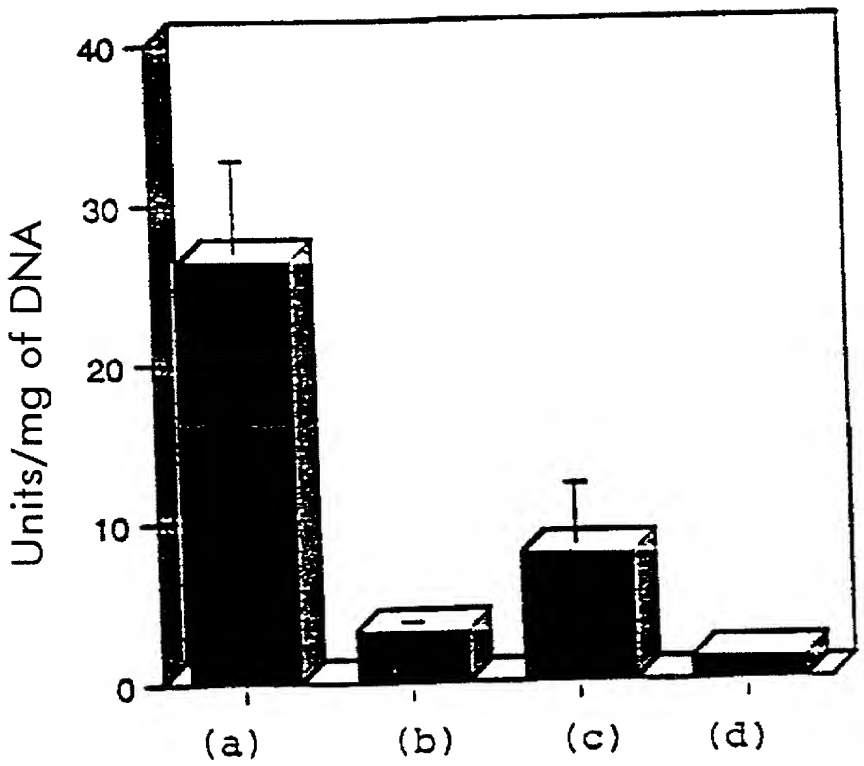
14. The composition as claimed in claim 13, characterized in that the following components are included:

- a solution suitable for alkaline lysis of biological sample material,
- a salt solution containing 1 to 6 M potassium acetate,
- an alcoholic solution containing 0.5 to 10% (w/v) SDS in 100% strength isopropanol and
- a silica gel-like support material.

15. The composition as claimed in claim 13 or 14, characterized in that the support material included is a suspension of silicon dioxide.

16. The use of potassium acetate for isolating, purifying and/or separating endotoxin-free nucleic acids and/or oligonucleotides or nucleic acids and/or oligonucleotides with reduced endotoxin content from and of, respectively, a pre-purified biological sample.

Figure 1



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Docket No. _____

ARENT FOX KINTNER PLOTKIN & KAHN, PLLC
Nikaido, Marmelstein, Murray & Oram Intellectual Property Group

Declaration For U.S. Patent Application

As a below named inventor, I hereby declare that:
My residence, post office address and citizenship are as stated below my name.
I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled
(Insert Title) Method for preparing endotoxin-free nucleic acids or nucleic acids with reduced endotoxin content and the use thereof
the specification of which is attached hereto unless the following box is checked:

☒ was filed on January 26, 2000 as PCT International Application
Number PCT/EP 00/00564 and was amended on ----------
and/or was filed on July 26, 2001 as United States Application
Number 09/890,202 and was amended on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claim(s), as amended by any amendment referred to above.
I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56.
I hereby claim foreign priority benefits under 35 U.S.C. §119(a)-(d) or §365(b) of any foreign application(s) for patent or inventor's certificate, or §365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below any foreign application for patent or inventor's certificate or PCT International Application having a filing date before that of the application(s) for which priority is claimed:

(List prior foreign applications. See note A on back of this page)	(Number)	(Country)	(Day/Month/Year Filed)	Priority Claimed
	199 03 507.5	DE	Jan 29, 1999	☒ Yes ☐ No
				☐ Yes ☐ No
				☐ Yes ☐ No

I hereby claim the benefit under 35 U.S.C. §119(c) of any United States provisional application(s) listed below.

(Application Number) (Filing Date)

(Application Number) (Filing Date)

(See Note B on back of this page) ☐ See attached list for additional prior foreign or provisional applications.

I hereby claim the benefit under 35 U.S.C. §120 of any United States application(s) or §365(c) of any PCT International application(s) designating the United States of America listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior application(s) (U.S. or PCT) in the manner provided by the first paragraph of 35, U.S.C. §112, I acknowledge the duty to disclose information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application.

(List prior U.S. Applications or PCT International applications designating the U.S.)	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
	(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

And I hereby appoint as principal attorneys: Robert B. Murray, Reg. No. 22,980; Charles M. Marmelstein, Reg. No. 25,895; George E. Oram, Jr., Reg. No. 27,931; Douglas H. Goldhush, Reg. No. 33,125; David T. Nikaido, Reg. No. 22,663; Monica Chin Kitts, Reg. No. 36,105; Richard J. Berman, Reg. No. 39,107; King L. Wong, Reg. No. 37,500; James A. Poulos, III, Reg. No. 31,714; Patrick D. Muir, Reg. No. 37,403; Murat Ozgu, Reg. No. 44,275; Bradley D. Goldizen, Reg. No. 43,637, N. Alexander Nolte, Reg. No. 45,689 and Robert K. Carpenter, Reg. No. 34,794.

Please direct all communications to the following address: ARENT FOX KINTNER PLOTKIN & KAHN, PLLC
1050 Connecticut Avenue, N.W., Suite 600
Washington, D.C. 20036-5339
Telephone No. (202) 857-6000; Facsimile No. (202) 638-4810

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further, that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

(See Note C on back of this page)

Full name of sole or first inventor GRIMM Stefan
Inventor's signature X _____ X 23.8.01 Date
Residence Richard-Riemer-Schmid-Allee 7, 81241 München, DE
Citizenship German
Post Office Address same as above

Full name of second joint inventor, if any NEUDECKER Frank

Inventor's signature × [signature] × 23.08.04 Date

Residence Treitschkestraße 18, 80992 München, DE DEX

Citizenship German

Post Office Address same as above

Full name of third joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

Full name of fourth joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

Full name of fifth joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

Full name of sixth joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

Full name of seventh joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

Full name of eighth joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

Full name of ninth joint inventor, if any _____

Inventor's signature _____ Date

Residence _____

Citizenship _____

Post Office Address _____

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Assistant Commissioner for Patents
Washington, DC 20231

GENERAL APPOINTMENT OF REPRESENTATIVE FOR U.S. PATENT AND TRADEMARK OFFICE MATTERS

The undersigned applicant or assignee hereby appoints D. Michael Young, Reg. No. 33,819, Brent A. Harris, Reg. No. 39,215, Richard T. Knauer, Reg. No. 35,575, Kenneth J. Waite, Reg. No. 45,189, Marilyn L. Amick, Reg. No. 30,444, and Michelle Neff, Reg. No. 47,817, all of Roche Diagnostics Corporation, 9115 Hague Road, P.O. Box 50457, Indianapolis, Indiana 46250, Telephone No. (317) 845-2000, and Jill Lynn Woodburn, Reg. No. 39,874 of The Law Office of Jill L. Woodburn, L.L.C., 6633 Old Stonehouse Drive, Newburgh, Indiana 47630-1785, Telephone No. (812) 842-2660:

to prosecute and transact all business on its behalf before the United States Patent and Trademark Office in connection with any U.S. patent assigned to it and any U.S. patent application filed by it or on its behalf and to receive payments on its behalf.

Signed this 11th day of April, 2001 at Mannheim, Germany.

Roche Diagnostics GmbH	Roche Diagnostics GmbH
M. Jung	Anton Silber
Signature	Signature
Dr. Michael Jung	Dr. Anton Silber
Print Name	Print Name
Senior Director	Director
Position or Title	Position or Title

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